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14. ABSTRACT

This project addressed the role of RUNX1 and ID1 transcription factors in regulating the biology of myofibroblast progenitor cells in the tumor microenvironment of prostate cancer. Task 1 used novel 3D organoid and co-culture models. We have found that RUNX1 is critical mediator of TGF-beta action in mesenchymal stem/progenitor cells. RUNX1 is critical for cell cycle progression and proliferation of progenitors. RUNX1 also limits differentiation to myofibroblasts and maintains proliferative status. TGF-beta mediated gene expression and the role of RUNX1 have been determined. Experiments to address ID1 were changed in year 2-3 to address p53 in the NCE period. RUNX1 and p53 were found to regulate cell proliferation but not to interact. Importantly, we also found that IL-1apha regulated cell differentiation. Task 2 focused on in vivo biology and the role of the ELF3 factor. RUNX1 knockdown appeared to limit proliferation of myofibroblasts in xenograft tumors. ELF3 was found to mediate IL-1alpha induction to an immune reactive phenotype. Together, these studies point to critical roles of RUNX1 and ELF3 in the genesis of tumor-promoting reactive stroma in prostate cancer.

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Introduction:

This project focused on evaluating mechanisms through which the RUNX1 and ID1 transcription factors regulate prostate stromal cell expansion and differentiation to tumor-promoting myofibroblasts. The proposed studies were focused on two Tasks. Task 1 is focused on RUNX1 and ID1 mechanisms of action in mesenchymal stem cells (MSCs) and myofibroblasts. Task 2 is focused on their roles in myofibroblast biology during regulation of tumorigenesis. We have shown previously that TGF-β1 is overexpressed in prostate carcinoma cells and is a key factor in regulating the formation of reactive stroma in the tumor microenvironment [1, 2]. We have also reported that reactive stroma is tumor promoting in xenograft models [3-6]. Our previous Annual Reports have described our model systems and key data gained during the project. Our studies to date have isolated and characterized several human prostate stromal cell lines derived from normal adult cadaver donors. We have determined that these cells are multi-potent tissue-resident mesenchymal stem cells. Moreover, we determined that RUNX1 is the key transcription factor that is necessary for the proliferation and expansion of mesenchymal stem cells to form a biologically effective mass of myofibroblasts, the principal cell type in reactive stroma. These data were recently published in the Proceedings of the National Academy of Sciences (PNAS) [7]. This publication is attached. This work represents a major advancement in the field. The work is the first report of mesenchymal stem cells isolated from the normal human prostate gland, the development of a novel 3D organoid culture model, and the identification of the RUNX1 transcription factor as a mediator of TGF-beta action in mesenchymal stem cells. Interest in this manuscript also led to an invited published commentary in PNAS that highlights this work [8]. This key publication essentially contains data that is addressed in both Task 1 and Task 2 of the project as summarized below.

Keywords:

Reactive stroma; mesenchymal stem cells, myofibroblasts, tumor microenvironment, RUNX1 transcription factor; ID1 DNA-binding factor

Overall Project Summary:

Task 1: "Task one will be to conduct experiments to determine the mechanisms through which RUNX1 and ID1 regulate differentiation of mesenchymal stromal cells to myofibroblasts and the subsequent interactions of myofibroblasts with prostate cancer cells using the 3D organoids to evaluate specific endpoints. Work in this Task will also address the role and mechanisms of these transcription factors in promotion of androgen-regulated gene expression in prostate cancer cells induced by myofibroblast/CAFs." Timeline: This Task was proposed to take the first 24-30 months of the project to complete.

> "Description: These experiments are outlined in Specific Aim 1. This Aim will use the novel three dimensional (3D) organoid model that recombines engineered human prostate cancer cells with human prostate mesenchymal myofibroblast progenitor cells in a 3D organoid in fully defined and androgen-free media conditions.
>
> The experiments will test the role of active TGF-β1 expressed by the engineered prostate cancer cells and will test whether these effects are mediated via the activity and downstream pathways regulated by either RUNX1 or ID1 transcription factors expressed in the myofibroblast progenitor cells. Experiments will also test the whether silencing of RUNX1 or ID1 will affect how myofibroblasts induce androgen regulated gene

expression in human prostate cancer cells in androgen-free media conditions and the key mechanisms that regulate this biology."

Task 1 Progress: All of the data generated in Task 1 was reported in our PNAS publication [7] attached in the Appendix. For Task 1 studies we developed a novel 3D organoid culture system (outlined in Figure 2 of the attached publication) to evaluate interactions between prostate carcinoma cells (LNCaP) engineered to express constitutively active TGF-β1 and human prostate stromal cells as proposed. We used this 3D model system and indirect co-culture systems to address the role of RUNX1 and ID1 using both human prostate mesenchymal stem cells (hpMSCs) (lines HPS-19I and HPS-33Q described in the Annual Report two years ago and in the original application) as well as human marrow-derived MSCs as positive controls. Each line was multipotent and could be induced to osteoblastic, chondroblast, nerves, or myofibroblast differentiation with specific media formulations (Figure 1 of attached publication). This pattern of differentiation potential is a key characteristic of MSCs. Each cell line exhibited MSC-specific cell surface markers as shown by flow cytometry analysis. Each line expressed genes that have been reported previously in MSCs. As determined by flow cytometry, the HPS-19I, HTS-33Q, and MSCs (Lonza) were each positive for CD13, CD29, CD105, CD73, CD44, and CD90. All were negative for CD106, and STRO-1. Together with the multipotency studies, this work shows that the human prostate-derived mesenchymal stem/progenitor cells exhibit properties consistent with marrow-derived mesenchymal cells. This was an important conclusion to be drawn from the Task 1 studies .

Laser capture of stromal cells from the 3D model was used to evaluate TGF-β1 induced gene expression. TGF-β1 / LNCaP cells induced expression of α-SMA (ACTA2), tenascin-C (TNC), FAP, collagen I, FGF-2, vimentin, and connective tissue growth factor (CTGF) in the responding hpMSC cells relative to control conditions (Figure 2 of attached publication). Together with immunohistochemistry confirmation of protein expression for some genes, these data confirmed that TGF-β1 was inducing differentiation to prototypical myofibroblasts. Flow cytometry separation of cells showed similar results. These studies showed that TGF-β1 expressing LNCaP cells induced expression of *WNT5A, WNT11, TGFB1, IGF1, RUNX1*, and inhibited expression of *CXCL12* and *BMP2*.

Subsequent experiments were conducted as proposed, to knockdown RUNX1 and ID1 or overexpress them to assess their function in hpMSCs (Figure 3 of attached publication). Two siRNAs were selected based on high (approximately 90%) knockdown efficiency after screening. Knockdown of RUNX1 resulted in elevated α-SMA and tenascin-C expression in hpMSCs. These data initially were opposite from what we had predicted as we reasoned that RUNX1 would likely mediate the effects of TGF-β1 on induction of α-SMA and tenascin-C. Elevated expression of RUNX1 did not seem to affect the biology relative to wild type expression levels.

These data suggested to us that RUNX1 may function to maintain a proliferative rate in the hpMSCs during their commitment to myofibroblasts. In all cell lines, including control marrow-derived MSCs, knockdown of RUNX1 induced a complete cessation of cell proliferation and an exit from the cell cycle as shown with flow cytometry studies (Figures 4 & 5 of attached publication). Inhibited expression of some cyclins and elevated expression of some inhibitors were shown and this was consistent with exit from cell cycle. These data represented highly significant findings. These data suggest that altering RUNX1 levels could be used to alter proliferation rate of developing reactive stroma from mesenchymal stem cells. Since the expansion of reactive stroma is associated with a more rapidly progressing prostate cancer and

with a higher likelihood of death due to prostate cancer, we proposed that this finding is very important (see Figure 6 model in attached publication).

The role of ID1 was addressed using similar approaches. Elevated expression of ID1 in preliminary experiments suggests that cells are stimulated in proliferation. If this is verified, then ID1 may also play a key role in regulating MSC proliferation and eventual differentiation to myofibroblasts in the reactive stroma tumor microenvironment. As we reported in the previous Annual Report, knockdown of ID1 did not produce any alteration in cell proliferation or apparent phenotype. We discovered that ID3 can modulate many of the effects as ID1. Hence, genetic ablation of ID family activity was deemed infeasible upon further study this past period. This was outlined in our previous Progress Report and NCE request.

To summarize our data, we believe RUNX1 functions to permit continued stemness and proliferative status in hpMSCs during induction by TGF-β1 to reactive stroma myofibroblasts. Our hypothesis is that TGF-β1 stimulates RUNX1 expression in these cells as they undergo transient amplification to reach a critical mass of reactive stroma myofibroblasts (Figure 6 of attached publication). It is possible that RUNX1 permits an asynchronous pattern of cell division. The ability to form a biologically effective mass of myofibroblasts is an important concept in wound healing and in the tumor microenvironment, which mimics wound healing in several regards. Accordingly, we feel that the TGF-β1 / RUNX1 signaling axis is critically important in regulating the mass of myofibroblasts in the tumor microenvironment. We have reported previously that the volume (mass) of reactive stroma in prostate cancer is associated with a more rapid progression and with prostate cancer associated death [9, 10]. Others have substantiated this finding in subsequent publications [11]. It follows that targeting the RUNX1 axis and/or the biology regulated by RUNX1 could represent a novel therapeutic approach in treating prostate cancer via altering the biology of the tumor microenvironment.

Task 1 Progress During the NCE Period: We have done additional experiments during the NCE period that directly relate to the activity of RUNX1 in human prostate MCSs. These studies have identified another important transcription factor that regulates the proliferation and differentiation of the MSCs. As disclosed in our previous Progress Reports and NCE request, we had experienced difficulty in addressing the actions of ID1 as originally proposed and have therefore addressed other potential transcription factors that may affect gene expression and differentiation of our prostate MSCs. Several observations suggested that the IL-1α interleukin may be an important upstream factor that regulates a differentiation profile of MSCs during wound repair. We set out to identify if this occurs with our human prostate MSCs and what transcription factor may mediate these effects. We also wanted to see if these factors interact with RUNX1. Hence we addressed this issue during the NCE in collaboration with a study funded by another source. These studies found that indeed, IL-1α is a key cytokine that regulates the transit amplification (proliferation) of our human prostate MSCs and induced their differentiation to a novel fibroblast phenotype that we think is very important in the prostate cancer tumor microenvironment and in reactive stroma in BPH.

These studies showed that IL-1α was a potent inducer of human prostate MSCs to an immune reactive phenotype with a gene expression profile consisting of cytokines / chemokines (Figure 1). Expression of key factors were validated. Biological functions of these factors predicts these as would highly recruit neutrophils, MDSCs, and other immune cell types to the stromal microenvironment. Importantly, IL-1α upregulated ELF3 expression in prostate MSCs and identified it as a potential mediator of IL-1α actions. ELF3 (E74-like transcription factor 3) is a transcription factor, shown previously to regulate gene expression in vascular inflammation and

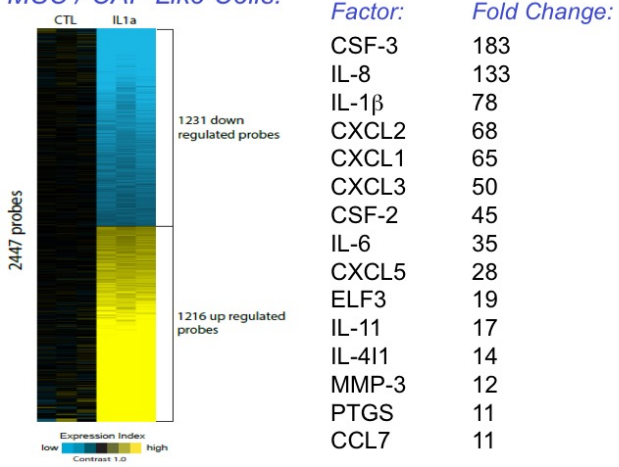

Figure 1. Expression of IL-1α induced immune regulatory factors by prostate MSC/CAFs in indirect 3D co-cultures with prostate epithelial cells expressing IL-1α.

other inflammatory responses. ELF3 has not been studied in MSC activation and differentiation. We showed that knockdown of ELF3 inhibited IL-1α induction of gene expression in these cells (Figure 2). Importantly, these studies also showed that IL-1α induced a differentiation of MSCs to an immune regulatory stromal phenotype that was not reversible to an MSC or a general myofibroblast, even when stimulated by TGF-beta. We examined this further and showed that when the MSCs were induced to differentiate to immune reactive CAF-like cells, they were refractory to TGF-β activities and could not be induced to become the typical myofibroblasts as studied in Task 1 studies.

Importantly, our studies on showed that knockdown of ELF3 in human prostate MSCs resulted in blocked cell proliferation (Figure 3). This is a similar effect to what we published for RUNX1. There is one paper that showed that RUNX1 and ELF3 interact to regulate expression of the angiopoietin gene. These results are very interesting and we plan to develop a future grant application to address how RUNX1 and ELF3 may differentially regulate MSC differentiation patterns to either an inflammatory fibroblast or to a matrix producing myofibroblast. Pathways regulated by both of these pathways could evolve as targets of opportunity based on these studies.

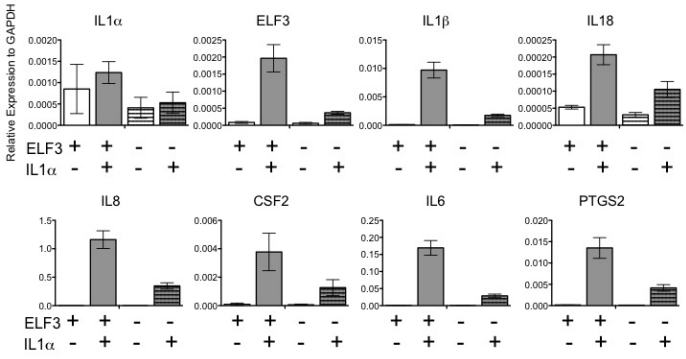

Figure 2. IL-1α induces cytokine gene expression in human prostate MSCs in an ELF3 dependent manner. ELF3 knockdown: ELF3-

Relative to Task 1 goals, although we were not able to address ID1 due to technical reasons, as stated in our Progress Reports and NCE requests, we were able to identify ELF3 as a transcription factor that is critical for proliferation / transit amplification of MSCs to become reactive stroma progenitors. As such, these studies were successful as they identified both RUNX1 and ELF3 pathways as targets of opportunity for future study to identify novel therapeutics.

Task 2: "Task two will be to evaluate the role of RUNX1 and ID1 in the promotion of prostate cancer tumorigenesis by reactive stroma myofibroblasts. These experiments will use DRS xenografts to address how silencing of RUNX1 and ID1 affects mechanisms of

myofibroblast/CAF differentiation and promotion of tumorigenesis, including expression of androgen-regulated genes." Timeline: This Task was proposed take months 6-36 to complete.

Task 2 Progress to Date: As part of the PNAS publication, we have also completed several in vivo xenograft studies as proposed in Task 2. We have been able to show that wild type HPS-19I cells differentiate to prototypical myofibroblasts in vivo when recombined with LNCaP cells and injected as a xenograft tumor (subcutaneously) in NCr *nu-nu* "nude" mice. In vivo xenografts were generated with RFP-labeled hpMSCs engineered with RUNX1 knockdown resulted in heterogeneous tumors. In general, these tumors exhibited greater staining intensity of α−SMA positive / RFP positive reactive stromal cells as compared to control tumors (Supplemental Figure S13) [7]. These tumors also exhibited reduced Ki67 staining in the reactive stroma suggesting a lower proliferation rate as well (Supplemental Figure S16). We have not yet constructed xenograft tumors with hpMSCs overexpressing RUNX1 or ID1.

The long-range significance of this study is the potential for exploiting the RUNX1 pathway therapeutically as a mechanism to attenuate the tumor promoting microenvironment.

Change in Approach during NCE and Reasons for Change: As outlined in the NCE request submitted previously, our data suggested RUNX1 effects may be balanced by p53 activity. We previously requested use of NCE funds to explore this avenue, as we believe may be more fruitful that trying tie in the activity of ID1. Below, is a quote from our approved NCE request.

"We have experienced delays in knocking down both ID3 and ID1 as reported in our previously approved Progress Report. We have also discovered, during the last project period, that RUNX1 and p53 may exhibit antagonistic roles in mediating mesenchymal stem cell (MSC) biology. Importantly, previous studies have shown that these transcription factors interact with each other. Since the overall goal of this study is to understand the mechanisms of RUNX1 actions in regulating MSC proliferation and differentiation to myofibroblasts in the tumor microenvironment, we believe that this new data is critically important. We request use of remaining funds to follow-up on this interaction as understanding the biology is very important to Task 1 and Task 2 goals. In particular, the NCE will permit us to complete part of Task 2 in vivo xenograft studies. We plan to examine the gain and loss of RUNX1 activity as originally proposed. In addition, we request use of remaining funds to also address the action of RUNX1 in a p53 null background. These studies will help us understand more completely, the pathways through which RUNX1 induces MSC biology (the overall goal of the project) and the specific role of p53 in these pathways. The data we have generated over the last year lead us to believe that these interactions are very important. We believe that data from a RUNX1 / p53 interaction model will serve the field in a relevant and significant manner."

"Conducting these experiments is important for the success of the proposed research program. First, it is important to understand the role of RUNX1 action in xenografts composed of prostate stromal MSC cells and cancer cells as proposed. Second, it is important to know what interacting transcription factors modulate RUNX1 activity. We now believe the p53 transcription factor is the key RUNX1 modulator. Hence, it is important to complete our project in order to define these pathways in vivo and complete our goals."

"We plan to use the remaining funds to conduct the Task 2 in vivo xenograft experiments. The only changes proposed would be to modulate the levels of p53 rather than ID1 in these studies, as our recent data points to p53 as the key interacting factor with RUNX1 that modulates MSC biology. We plan to use the DRS model as proposed."

Task 2 Progress During the NCE Period: As proposed, we conducted experiments to address the role of p53 in regulating RUNX1 during the NCE period. First, we showed that TGF-β1 did not elevate p53 levels, unlike RUNX1 (Figure 3). Of interest, knockdown of p53 resulted in a sustained level of Ki67 and at the same time stimulated expression of smooth muscle a-actin (ACTA2) (Figure 4A & 4B). We then showed that opposite to the effects of RUNX1, knockdown of p53, via siRNA stimulated cell proliferation (Figure 4C). Hence, these data were consistent with our hypothesis stated in the NCE request. This was confirmed using CRSPR/Cas for stable p53 knockout (Figure 4D). This led us to conclude that p53 functioned to limit expansion (transit amplification) of these cells and their differentiation to mature myofibroblasts. RUNX1 was shown the increase the proliferation of MSCs. Both seemed to repress differentiation status as measured by ACTA2 expression. Part of our hypothesis was based on the studies of Wu et al., who demonstrated a physical interaction of p53 with RUNX1 on target genes and this was verified by immunoprecipitation pull down Western blots using HCT116 colon cancer cells [12]. Accordingly, a major thrust of the studies and budget during the NCE period was to address whether this physical interaction existed between RUNX1 and p53 in our human prostate MCSs.

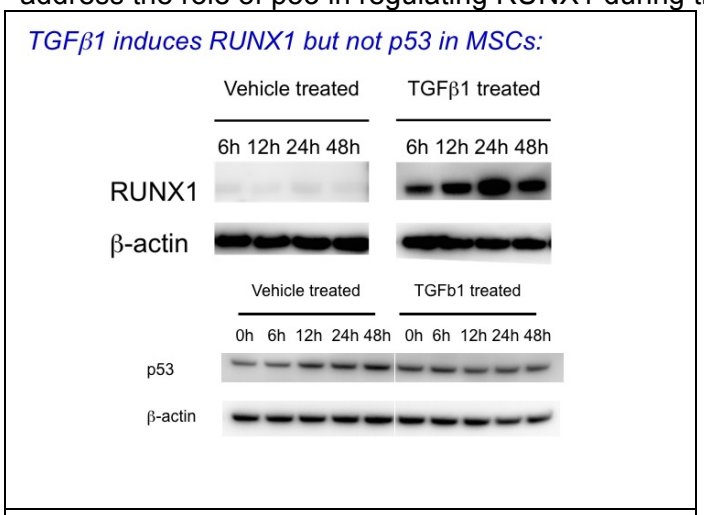

Figure 3. RUNX1 is regulated by TGF-β1, whereas p53 is not.

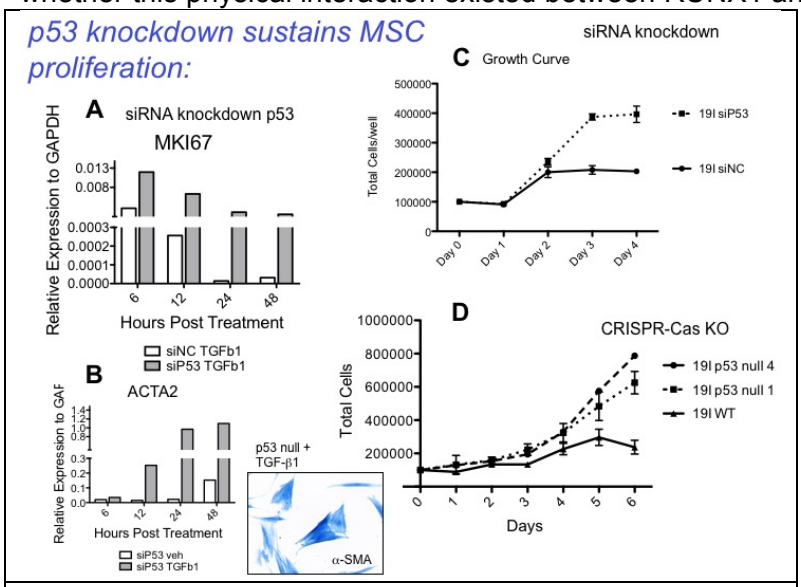

Figure 4. Knockdown of p53 in human prostate MSCs results in stabilized Ki67 (MKI67) and smooth muscle α-actin (ACTA2) (Panels A & B). This is associated with sustained proliferation (Panels C & D).

The Wu studies showed that both RUNX1 and p53 were recruited to p53 target genes and that RUNX1 stimulated p53 activity. It was concluded in the Wu et al study that RUNX1 is required for p53 activity in these cancer cells in response to DNA damage [12]. Of considerable interest, our work during the NCE showed that RUNX1 / p53 direct interactions do not exist, as determined by immunoprecipitation/Western pull down experiments, in human prostate mesenchymal stem cells in +/- DNA damage conditions or in vehicle or TGF-β1 treated conditions (Figure 5). As a positive control, we used the HCT116 colon cancer cells in +/- DNA damage conditions and we did see RUNX1 / p53 interaction in the HCT116 cells when they were subjected to DNA damage (Figure 5), thereby confirming the claims in the Wu publication.

These data were of much interest to us. There are several explanations and we hope to build upon the observations made during the NCE period to address some of the key questions that

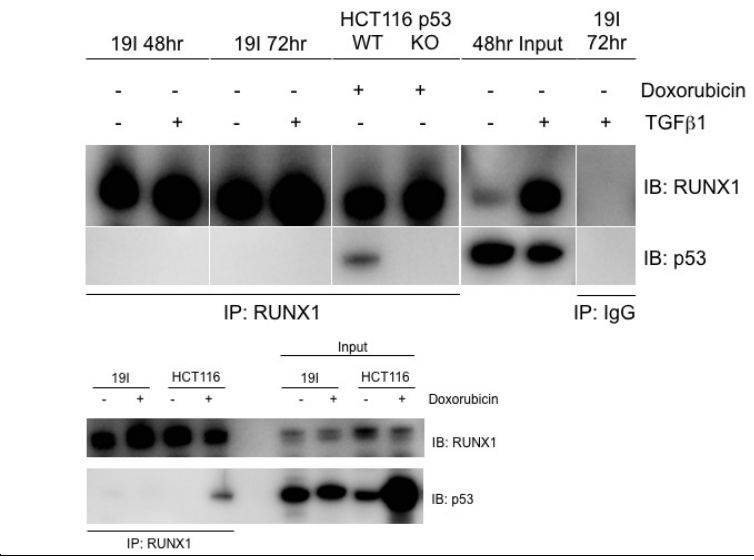

Figure 5. Immunoprecipitation and Western blotting reveals that RUNX1 and p53 directly interact in HCT116 cells upon DNA damage (+Doxorubicin) but do not interact in DNA damaged or control human prostate MSCs whether treated with TGF-β1 or not.

arise. Our data showed that RUNX1 functions to stimulate proliferation (transit amplification) of human prostate MCSs, whereas p53 seems to limit proliferation. In constrast, the studie by Wu suggested these factors work together to regulate p53 target genes. In this case RUNX1 is required for proper p53 function. The differences could be due to the fact that the HCT116 cells beign used in the Wu study were cancer cells of epithelial origin. Our cells, in contrast, are normal stromal mesenchymal stem cells. Hence differences in the type of cell, cancer vs normal or epithelium vs stromal (or both of these) might explain the differences. It could also be that when RUNX1 and p53 directly interact, RUNX1 has an agonist role, whereas when they are both active in the nucleus but do not interact (or are prevented from interacting), there is a antagonistic effect. We were restricted by time and budget to address these extended questions in the NCE period.

To summarize, we did demonstrate that the growth promoting activity of RUNX1 is indeed balanced by p53, whereas the differentiation inhibition is shared by both. However, our studies also showed that this activity occurs absent of direct physical interactions of these two factors. Addtitional CHIP-Seq experiments are now needed to address this question further and we hope to use the data generated from the NCE period to prepare both a manuscript and a grant application to address the extended questions in detail.

We were not able to complete the proposed in vivo studies owing to time, the remaining NCE budget, and our desire to better understand some cell and molecular mechanisms of both p53 and ELF3 transcription factors before embarking on expensive in vivo studies. ELF3 was identified as another key factor, whose significance to MSC activation and proliferation we did not yet fully comprehend when the NCE was requested.

Change in Personnel and Reasons for Change: No key change in Personnel from the previous Annual Report and NCE request. NCE funds were used primarily to support Linda Tran as outlined in the Previous Annual Report and NCE request. As this project was on NCE, Mr. Dang no longer provided an effort as was disclosed in the previous Progress Report. Ms. Tran is a senior graduate student and completed the work during the NCE period. Each of these changes were outlined previously in the Budget and Budget Justification submitted with the NCE request.

Key Research Accomplishments:

- Determination that human prostate derived HPS-19I and HPS-33Q are multipotent normal human prostate-derived mesenchymal stem/progenitor cells (hpMSCs). We have shown that these cells can be induced to differentiate to myofibroblasts, osteoblasts, chondrocytes, and nerve. This is a novel finding.
- Characterization of markers in these cells. The cells are positive for several prototypical markers of mesenchymal stem cells including CD44, CD90, CD13, CD29, CD73, CD105, and negative for CD106 and STRO-1.
- Further development and refinement of the 3D organoid model of recombined human prostate cancer cells with human prostate mesenchymal stem cells.
- Determination that elevated RUNX1 is mediated via TGF-beta signaling in human prostate-derived mesenchymal stem cells. TGF-beta signaling results in differentiation to a prototypical myofibroblast phenotype.
- Determination that RUNX1 is required for the transit amplification, proliferation and stem cell properties of these cells. Knockdown of RUNX1 results in cell cycle arrest and attenuated proliferation.
- Determination that ID1 elevates cell proliferation in more preliminary studies, however mechanisms have not yet been worked out. Inability to knockdown ID1 and ID3 activities precluded further investigation of specific mechanisms.
- Generation of an hypothesis that the transit amplification of hpMSCs is dependent on and regulated by RUNX1 and that this amplification is a key aspect of reactive stroma formation. The mass of reactive stroma in human prostate cancer is a predictive indicator of rate of progression and we have reported previously that this volume is predictive of men who will die from their prostate cancer. Accordingly, attenuation of the reactive stroma mass may be a method to reduce the rate of tumor growth.
- Publication of a manuscript focused on the role of RUNX1 in myofibroblast differentiation in Proceedings of the National Academy of Science (PNAS) [13]. This manuscript contains Task 1 proposed studies as well as experiments supported by other projects and some of the in vivo tumorigenesis studies proposed in Task 2 experiments. Manuscript Title: RUNX1 is essential for mesenchymal stem cell proliferation and myofibroblast differentiation (see attached in Appendix). This is a key publication in the field and is the first to isolate and characterize mesenchymal stem cells from the normal human prostate gland. Moreover, this was the first manuscript to delineate the role of the RUNX1 transcription factor in human mesenchymal stem cells. Accordingly, the impact of this publication is relevant to all human cancers, in addition to prostate cancer, and other disorders associated with mesechymal stem cells. A commentary attesting to this significance of this manuscript was published in the same issue [8].
- Generation of the hypothesis that elevated nuclear RUNX1 maintains hpMSCs in transit amplified state and that this is primarily tumor-promoting, whereas fully differentiated myofibroblasts may be more tumor promoting. This hypothesis was directly generated from the funded studies.
- Generation of key data and hypotheses that RUNX1 and p53 may reciprocally regulate MSC transit amplification potential and that p53 functions as an opposing mechanisms to regulate the extent of RUNX1-induced proliferation in human prostate MSCs.
- Determination that RUNX1 and p53 do not interact physically in human prostate MSCs, but do interact in HCT116 colon carcinoma cells as a positive control.
- Generation of an hypothesis that RUNX1 and p53 influence the biology of human MSCs via indirect interactions but not likely through direct interactions as transcription factors.

- Identification of IL-1α as a key upstream cytokine that regulates gene expression differentiation of human prostate MCSs to a immune reactive CAF-like fibroblast cell type.
- Identification and characterization of the ELF3 transcription factor as the mediator of IL-1α activity in human prostate MSCs.
- Identification and characterization of ELF3 transcription factor as necessary for human prostate MCSs to undergo proliferative expansion (transit amplication).
- Summary conclusion that the secreted factors TGF-β1 and IL-1α function to differentially regulate human prostate MSC activation, proliferation (transit amplification) to either myofibroblast (TGF-β1 regulated, matrix producing and contractile) or immune reactive fibroblasts (IL-1α regulated, cytokine producing, inflammation producing, potential immune suppression) differentiated phenotypes that are mutually exclusive.
- Summary conclusion that the RUNX1 and ELF3 transcription factors mediate the effects of TGF-β1 and IL-1α respectively in human prostate MSCs.
- Summary conclusion that RUNX1 and ELF3, along with their regulated pathways, are targets of opportunity for future therapeutic strategies and approaches to alter co-evolution of a tumor-promoting reactive stroma microenvironment in human prostate cancer.

Conclusions:

The studies supported by this project have had a major impact in our knowledge of the origin of myofibroblasts in the tumor microevnironment of human prostate cancer. Moreover, this work has identified RUNX1 as a key transcription factor that regulates mesenchymal stem cell biology in several ways to effect wound repair and reactive stroma formation in the tumor microenvironment. The work was the first to isolate hpMSCs, to develop 3D organoid models using these cells, and to identify the biological activities of RUNX1 as a TGF-β1 mediated downstream effector. In addition, this work has identified IL-1α as a regulator of hpMSC differentiation to an immune reactive CAF-like fibroblast and ELF3 as the transcription factor that mediates this response. This work helps us understand the tumor-regulatory nature of the reactive stroma microenvironment in prostate cancer. Since most all carcinomas have a similar reactive stroma [14], we expect our results will have an impact in other cancer fields. Moreover, since the mechanisms of reactive stroma formation in cancer is similar in many ways to the formation of normal and abnormal wound repair stroma [15], our studies will likely impact in the understanding of several fibroses that are characterized by having an abundance of myofibroblasts. Overall, data generated as a result of this project will directly impact strategies to target the reactive stroma microenvironment therapeutically for the treatment of prostate cancer. Understanding fundamental mechanism is an important first step in this approach.

Publications, Abstracts, and Presentations:

- Publication of a manuscript focused on the role of RUNX1 in myofibroblast differentiation in Proceedings of the National Academy of Science (PNAS) in 2014. This manuscript contains most of Task 1 proposed studies as well as experiments supported by other projects and some of Task 2 experiments. Manuscript Title: RUNX1 is essential for mesenchymal stem cell proliferation and myofibroblast differentiation (see attached in Appendix).
- Presentation by graduate student Woosook Kim at IMBS Program (formerly the CMB Program) graduate student seminar series. Seminar presented in March 2013, Rm M315, Baylor College of Medicine.

- Poster presentation by graduate student Woosook Kim at 2013 AACR Annual Meeting. April 6-10, 2013, Walter E. Washington Convention Center, Washington DC.
- Oral presentation by graduate student Woosook Kim at US-KOREA Conference (UKC2013, New York, NY., August 8 - 10, 2013,)
- Invited plenary Symposia presentation by Dr. Rowley at the Annual Endocrine Society Meeting, June 18, 2013, San Francisco, CA. Title: "Role of Reactive Stroma in Prostate Disease"
- Invited podium presentation by Dr. Rowley at the Prout's Neck Meeting (Sponsored by the Prostate Cancer Foundation) June 23, 2013, Lake Tahoe, NV. Title: "Genesis of Reactive Stroma and Prostate Cancer Progression".
- Invited podium presentation by Dr. Rowley at the Lost Pines Conference, November 13, 2013, The Virginia Harris Cockrell Cancer Research Center at Science Park, MD Anderson Research Park, Smithville Texas. Title: "Genesis of Reactive Stroma in Prostate Cancer: RUNX1 Mediates TGF-β Action".
- Invited podium presentation by Dr. Rowley at the Coffey-Holden Prostate Cancer Academy meeting sponsored by the Prostate Cancer Foundation. June 27, 2014, La Jolla, CA. Title: "Regulators of Reactive Stroma: Therapeutic Resistance Implications".
- Invited Seminar Presentation by Dr. Rowley at University of Texas Medical Branch, Galveston, TX. Department of Pharmacology and Toxicology. December 19, 2014. Title " The Genesis of Reactive Stroma in Cancer: Role of TGF-beta and RUNX1".
- Invited podium presentation by Dr. Rowley at the Coffey-Holden Prostate Cancer Academy meeting sponsored by the Prostate Cancer Foundation. June 26, 2015, La Jolla, CA. Title: "Tumor Stroma Evolution: RUNX1 as a Potential Therapeutic Target"
- Poster presentation by graduate student Linda Tran at the Graduate Program in Integrative Molecular and Biomedical Sciences annual retreat, March, 2014. Title: Role of IL-1α mediated inflammation during prostatic disease.
- Seminar presentation by graduate student Linda Tran at Integrative Molecular and Biomedical Sciences seminar series, March, 2014. Title: Role of IL-1α mediated inflammation during prostatic disease.
- Abstract and poster presentation by graduate student Linda Tran. Graduate Student Symposia, October, 2014, Baylor College of Medicine. Title: Role of p53 in myodifferentiation during prostatic disease progression.
- Seminar presentation by graduate student Linda Tran. Graduate Program in Integrative Molecular and Biomedical Sciences. January, 2015. Title: Role of p53 in myodifferentiation during prostatic disease progression.
- Poster presentation by graduate student Linda Tran at the Dan L Duncan Comprehensive Cancer Center Annual Symposium, January 2015. Title: TGF-β1 and RUNX1 affect p53 protein dynamics during myofibroblast differentiation.
- Poster presentation by graduate student Linda Tran at the Graduate Program in Integrative Molecular and Biomedical Sciences annual retreat, March 2015. Title: TGF-β1 and RUNX1 affect p53 protein dynamics during myofibroblast differentiation.
- Invited Seminar Presentation by Dr. Rowley at Louisiana State University School of Medicine, Shreveport, LA. November 2015. Title: Co-evolution of Reactive Stroma in Cancer: Tissue Repair Biology and Cancer Progression.
- Podium presentation by graduate student Linda Tran at the Graduate Program in Integrative Molecular and Biomedical Sciences annual retreat, March 2016. Title: ELF3 mediates the IL-1α induced inflammatory response in mesenchymal stem cells.
- Invited podium presentation by Dr. Rowley at the Fall 2016 SBUR Sympoisa, November 10-13, 2016. Title: Stromal Cell Plasticity and Reprogramming in Prostate Disease.

Inventions, Patents and Licenses: None

Reportable Outcomes:

- Derivation of HPS-19I and HPS-33Q cells with RUNX1 knockdown.
- Generation of HPS-19I and HPS-33Q cells with elevated RUNX1 expression.
- Generation of HPS-19I cells with knockdown of ID1
- Generation of HPS-19I cells with elevated ID1 expression.
- Generation of a database of genes regulated in human prostate mesenchymal/stem cells stimulated by TGF-beta1 during differentiation to reactive stroma myofibroblasts.
- Derivation and optimization of several co-culture model systems including a three-dimensional model with recombined human prostate cancer cells with human prostate mesenchymal stem cells
- Human prostate derived HPS-19I and HPS-33Q represent multipotent normal human prostate-derived mesenchymal stem/progenitor cells (hpMSCs). We have shown that these cells can be induced to differentiate to myofibroblasts, osteoblasts, chondrocytes, and nerve. These data are included in our PNAS manuscript in press.
- These cells are positive for several prototypical markers of mesenchymal stem cells including CD44, CD90, CD13, CD29, CD73, CD105, and negative for CD106 and STRO-1.
- Elevated RUNX1 is mediated via TGF-beta signaling in human prostate-derived mesenchymal stem cells. TGF-beta signaling results in differentiation to a prototypical myofibroblast phenotype.
- Development of Western blots to assess RUNX1 protein levels.
- Generation of HPS-19I cells with p53 knockdown using CRISPR-Cas system.
- Generation of HPS-19I cells with p53 knockdown using siRNA system.
- Development of Western blots to assess p53 protein levels.
- Generation of a database with IL-1α induced gene expression profile from HPS-19I cells.
- Generation of HPS-19I cells with knockdown of ELF3.
- Generation of data showing IL-1α can irreversible induced growth and differentiation of HPS-19I cells to an immune reactive phenotype via the IL-1α/ELF3 pathways.

Other Achievements:

- The work on RUNX1 and ID1 in this project represented the thesis research project for the doctoral training of Woosook Kim, who successfully defended her thesis April 3, 2014. As indicated in the previous Annual Report, another student joined the laboratory, Ms. Linda Tran, who continued the project for year 03 and continued this during the NCE period. This extended work on p53 and IL-1α/ELF3 will form the basis of her thesis project.

Personnel Receiving Pay on this Project:

David R. Rowley, Ph.D. (PI,) Baylor College of Medicine
Woosook Kim, Ph.D. (Graduate Student) Graduated 2015, Baylor College of Medicine
Linda Tran (Current Graduate Student), Baylor College of Medicine
Truong Dang (Research Assistant), Baylor College of Medici

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Appendices:

1. Manuscript (PNAS) entitled "RUNX1 is essential for mesenchymal stem cell proliferation and myofibroblast differentiation". Proc Natl Acad Sci U S A, 2014. **111**(46): p. 16389-94.

SEE COMMENTARY

RUNX1 is essential for mesenchymal stem cell proliferation and myofibroblast differentiation

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Edited by Owen N. Witte, Howard Hughes Medical Institute, University of California, Los Angeles, CA, and approved September 22, 2014 (received for review April 18, 2014)

Myofibroblasts are a key cell type in wound repair, cardiovascular disease, and fibrosis and in the tumor-promoting microenvironment. The high accumulation of myofibroblasts in reactive stroma is predictive of the rate of cancer progression in many different tumors, yet the cell types of origin and the mechanisms that regulate proliferation and differentiation are unknown. We report here, for the first time to our knowledge, the characterization of normal human prostate-derived mesenchymal stem cells (MSCs) and the TGF-β1–regulated pathways that modulate MSC proliferation and myofibroblast differentiation. Human prostate MSCs combined with prostate cancer cells expressing TGF-β1 resulted in commitment to myofibroblasts. TGF-β1–regulated runt-related transcription factor 1 (RUNX1) was required for cell cycle progression and proliferation of progenitors. RUNX1 also inhibited, yet did not block, differentiation. Knockdown of RUNX1 in prostate or bone marrow-derived MSCs resulted in cell cycle arrest, attenuated proliferation, and constitutive differentiation to myofibroblasts. These data show that RUNX1 is a key transcription factor for MSC proliferation and cell fate commitment in myofibroblast differentiation. This work also shows that the normal human prostate gland contains tissue-derived MSCs that exhibit multilineage differentiation similar to bone marrow-derived MSCs. Targeting RUNX1 pathways may represent a therapeutic approach to affect myofibroblast proliferation and biology in multiple disease states.

myofibroblast | MSC | RUNX1 | TGF-β1 | reactive stroma

Myofibroblasts are key mediators of homeostasis-associated biology in reactive stroma associated with tissue repair, fibrosis, and the tumor microenvironment. However, the specific cell types of origin and the mechanisms that regulate their activation, proliferation, and differentiation are not understood. Accordingly, identifying the niche and origins of myofibroblasts and pathways that regulate their proliferation and commitment to myofibroblasts is important to developing antifibrotic and anticancer therapeutic approaches designed to target the evolution and proliferation of myofibroblasts.

Adult mesenchymal stem cells (MSCs) are defined as multipotent stromal cells capable of self-renewal and differentiation to cartilage, bone, and adipose tissues (1). Studies have primarily addressed the biology of marrow-derived MSCs and far less is understood about putative tissue-resident MSCs and how they contribute to the biology of their tissue of residence. Recent evidence from mouse models implicates tissue-resident MSCs in repair processes of disrupted local tissue homeostasis, suggesting that they are a critical component in wound repair, fibrosis, cardiovascular disease, and in the tumor microenvironment (2). Each of these is associated with evolution of reactive stroma with expression of repair-centric genes. However, very little is known about adult human tissue-resident MSCs and the mechanisms that regulate their proliferation and lineage commitment to local reactive stroma or fibrotic tissue.

Reactive stroma is dynamic and responds rapidly to emerging situations to restore disrupted homeostasis (3, 4). Reactive stroma is found in most cancers and is typified by the coevolution of myofibroblasts (5–7). Importantly, the volume of reactive stroma relative to cancer is predictive of the rate of cancer progression in several tumor types (8–15). Furthermore, in vivo modeling has shown that reactive stroma is tumor-promoting (16–19). The myofibroblast is the principal cell type in reactive stroma, and transforming growth factor beta 1 (TGF-β1) is a potent inducer of myofibroblast differentiation (3, 20). TGF-β1 is overexpressed in most human cancers, including prostate cancer (2, 7). However, the cell types of origin and mechanisms that regulate their proliferation and differentiation to myofibroblasts remain unknown.

To address tissue-resident MSCs in the prostate gland and their potential induction to myofibroblasts, we have evaluated whether tissue-associated MSCs reside in normal adult human prostate tissue, whether they are induced to become myofibroblasts by TGF-β1, and the key mechanisms that regulate their proliferation. We report here for the first time, to our knowledge, the isolation of cells with prototypical MSC properties derived from normal adult human prostate gland. These cells exhibit multipotent differentiation similar to bone marrow-derived MSCs and form typical reactive stroma myofibroblasts under TGF-β1 regulation. Importantly, we have identified runt-related transcription factor 1

Significance

Recruitment, proliferation, and differentiation of myofibroblasts are common in many disease states. Mechanisms that regulate proliferation and differentiation are poorly understood, although TGF-β is a key inducer of differentiation. Here, we report, for the first time to our knowledge, that runt-related transcription factor 1 (RUNX1) regulates mesenchymal stem cell (MSC) biology and progenitor cell commitment to myofibroblasts. In this work, we describe the first identification, to our knowledge, of tissue-resident MSCs from adult normal human prostate gland and the role of these MSCs as myofibroblast precursors. We also pinpoint the role of RUNX1 in regulating proliferation and differentiation in both marrow-derived and tissue-resident MSCs. Perturbation of RUNX1 activity may provide insights for developing antifibrotic and anticancer therapies via targeting the reactive stroma microenvironment.

Author contributions: W.K., K.S.C., and D.R.R. designed research; W.K., D.A.B., R.S.M., L.L.T., and F.Y. performed research; W.K., S.J.R., and D.R.R. analyzed data; and W.K. and D.R.R. wrote the paper.

The authors declare no conflict of interest.

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See Commentary on page 16238.

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(RUNX1) as a key transcription factor that is required for MSC/progenitor cell proliferation and that functions to moderate myofibroblast differentiation.

Results

Prostate-Derived CD44$^+$CD90$^+$ Cells Are Functional Mesenchymal Stem Cells. Fresh tissue cores from the peripheral zone of normal human prostate glands were obtained from 19-y-old (HPS-19I cells) and 33-y-old (HPS-33Q cells) cadaver donors with no prostate gland histopathology and explants cultured in a selective medium and protocol optimized for the culture of fetal rodent urogenital sinus mesenchyme (21). The resulting monolayers were evaluated for phenotypic markers and biology. Both HPS-19I and HPS-33Q cells exhibited spindle-shaped fibroblast-like morphology (Fig. 1*A*) and were capable of long-term culture for more than 25 passages, indicating a long-term growth potential. These cells exhibited density-dependent inhibition of cell growth when reaching more than 70% confluency, which restricted subsequent passage efficiency and differentiation potential. Both HPS-19I and HPS-33Q cells exhibited a normal diploid karyotype and lacked chromosomal aberrations, including translocations as indicated by spectral karyotyping (*SI Appendix*, Fig. S1). Flow cytometry showed that both cell lines were positive for CD44 and CD90 (Fig. 1*B*). To assess spatial distribution of CD44$^+$CD90$^+$ cells in prostate tissue, a tissue array with 32 normal human prostate tissue cores was evaluated via multispectral immunohistochemistry (IHC). Distinct clusters of CD44$^+$CD90$^+$ dual positive cells and individual cells were localized in the stromal compartment (Fig. 1*C*). In addition, dual positive immunoreactivity was observed in small vessels of the microvasculature and nerves. Although many fields exhibited a density of 1% or lower, the average density was 2.83% of total cells (*SI Appendix*, Fig. S2).

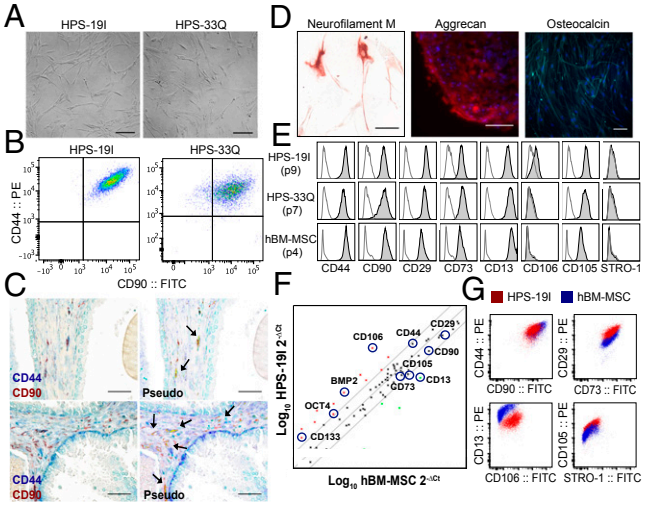

Fig. 1. CD44$^+$CD90$^+$ stromal cells derived from normal human prostate are mesenchymal stem cells. (*A*) Cell morphology. (Scale bars, 100 μm.) (*B*) Flow cytometric analysis of HPS cell lines. (*C*) Immunostaining of normal human prostate tissue for CD44 (blue), CD90 (red), and nuclei (green). Multispectral deconvolution microscopy revealed localization of CD44$^+$CD90$^+$ cells ("Pseudo" yellow, arrows) in the stroma. (Scale bars, 100 μm.) (*D*) Multilineage differentiation potential of HPS-19I cells. Neurogenesis is shown by neurofilament M staining ("Neurofilament M"). (Scale bars, 100 μm.) Chondrogenesis by aggrecan accumulation ("Aggrecan"). (Scale bars, 20 μm.) Osteogenesis by osteocalcin production ("Osteocalcin"). (Scale bars, 20 μm.) (*E*) Flow cytometric analysis of cell lines HPS-19I, HPS-33Q, and hBM-MSCs. "p" denotes passage. The open and gray plots represent the isotype control and the specific antibody indicated, respectively. (*F*) Similar gene expression profiles of HPS-19I cells compared with hBM-MSCs. (*G*) Flow cytometric analysis revealed that HPS-19I cells and hBM-MSCs share expression of MSC surface markers.

Owing to the expression of MSC-associated markers, long-term passage potential, and diploid status, we addressed whether these cells exhibit multipotent MSC characteristics. To address multilineage differentiation potential, HPS-19I and HPS-33Q cells were evaluated relative to human bone marrow-derived MSCs (hBM-MSCs). HPS-19I cells were induced to differentiate into neurogenic, chondrogenic, and osteogenic lineages (Fig. 1*D*), but were restricted from adipogenic differentiation. hBM-MSCs differentiated into all four lineages (*SI Appendix*, Fig. S3*A*). HPS-33Q cells could be induced to chondrocytes (*SI Appendix*, Fig. S3*B*), whereas osteogenic induction was less clear. Expression profiles of MSC-specific markers and stem cell markers in HPS-19I cells were congruent with profiles in hBM-MSCs (Fig. 1*F*). Moreover, flow cytometry revealed that the cell lines HPS-19I, HPS-33Q, and hBM-MSCs were each positive for CD44, CD90, CD13, CD29, CD73, and CD105, the most commonly reported human MSC markers, and were low for CD106 and STRO-1 (Fig. 1 *E* and *G*). Based on specific morphology, plastic adherence, multipotency, transcriptome, and cell-surface protein profiles of HPS-19I and HPS-33Q cells, we hereafter refer to these cells collectively as human prostate-derived mesenchymal stem cells (hP-MSCs).

Carcinoma hP-MSC Cell Interactions in 3D Organoid Cultures. A 3D organoid coculture system in defined medium that permitted the study of cell–cell interactions and differentiation potentials was developed (Fig. 2*A*). A combination of LNCaP human prostate carcinoma cells with hP-MSCs resulted in free-floating self-organizing organoids exhibiting a central core of hP-MSCs and an outer mantel of LNCaP (Fig. 2*B*). Organoids constructed with LNCaP and hBM-MSCs exhibited an identical phenotype (*SI Appendix*, Fig. S4*A*). IHC of 3D organoids showed that the core of hP-MSCs was positive for CD44 and CD90 and that the LNCaP mantel was positive for androgen receptor (AR) (Fig. 2*C*). LNCaP or hP-MSCs seeded alone also self-organized as a free-floating 3D organoid (*SI Appendix*, Fig. S4*A*) and could be indirectly cocultured with other cells as monolayers. Combination of LNCaP and hP-MSCs inoculated as s.c. xenografts in nude mice exhibited a similar self-organization in vivo with typical carcinoma foci and adjacent stroma (Fig. 2*B*; *SI Appendix*, Fig. S4*B*).

To investigate the function of TGF-β1 and hP-MSCs in the genesis of reactive stroma, hP-MSCs were cocultured with LNCaP engineered to express constitutively active TGF-β1 (*SI Appendix*, Fig. S6*A*). Organoids constructed with TGF-β1–expressing LNCaP exhibited elevated immunoreactivity for α-smooth muscle actin (α-SMA) and tenascin-C in hP-MSCs relative to control (Fig. 2*D*). Both are prototypical markers of reactive stroma myofibroblasts (7). Tenascin-C was predominantly localized in stroma immediately adjacent to epithelial cells, similar to patterns noted in human prostate disease (7). In addition, hP-MSCs exhibited increased immunoreactivity for both vimentin and fibroblast activation protein (FAP) under TGF-β1–stimulated conditions (*SI Appendix*, Fig. S5*A*). Furthermore, in vivo xenografts (day 11) generated with hP-MSCs combined with LNCaP resulted in a central core of α-SMA–positive myofibroblasts (*SI Appendix*, Fig. S4*C*). Labeling of the hP-MSCs via expression of RFP marker protein showed RFP and α-SMA dual positive cells in combined xenografts (day 10) (*SI Appendix*, Fig. S13).

TGF-β1–Induced Gene Expression Profiles of hP-MSCs Exhibit a Reactive Stroma Myofibroblast Signature. To identify gene expression profile alterations, we analyzed indirect cocultures of hP-MSC and engineered LNCaP organoids (Fig. 2*A*). ELISA analysis confirmed that active TGF-β1 was observed only in cocultures made with TGF-β1–expressing LNCaP (*SI Appendix*, Fig. S6*B*). hP-MSCs cocultured with LNCaP expressing TGF-β1 exhibited an increased expression of reactive stroma genes such as *TNC*, *ACTA2*, *FAP*, and *COL1A1* and showed distinct gene profiles including genes encoding cell-surface markers and growth factors

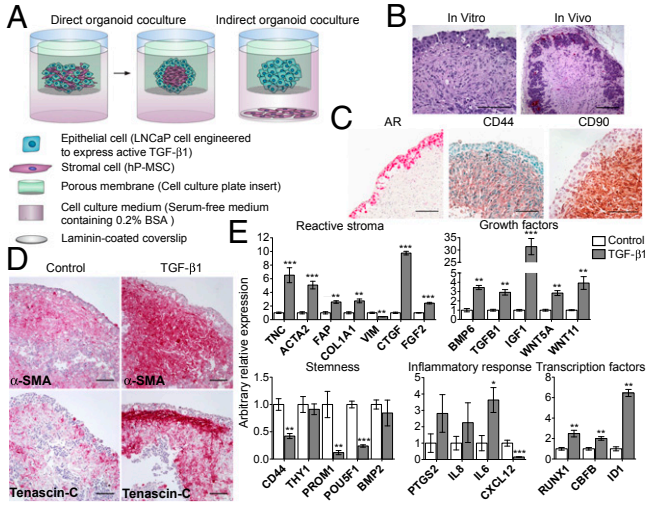

Fig. 2. hP-MSCs (HPS-19I) self-organize with LNCaP cells, and paracrine TGF-β1 drives hP-MSC differentiation into myofibroblasts with a reactive stroma signature. (*A*) Schematic diagram of 3D organoid coculture. LNCaP cells engineered to express active TGF-β1 were cultured as organoids on inserts. hP-MSCs were cocultured with engineered LNCaP cells either in direct contact or on laminin-coated coverslips in the bottom chamber. (*B*) HPS-19I cells self-organize with LNCaP cells in vitro and in vivo. Note that recombined 3D organoids exhibit a core of hP-MSCs surrounded by an outer layer of LNCaP cells. (*C*) Immunostaining of AR and CD44/CD90 in 3D organoids indicates a periphery of LNCaP cells and a core of hP-MSCs, respectively. (*D*) IHC analysis of α-SMA and tenascin-C in 3D organoids consisting of hP-MSCs and engineered LNCaP cells. Note the increase in both markers in hP-MSCs when cocultured with LNCaP cells expressing TGF-β1. (*E*) Gene expression analysis of markers for reactive stroma and stemness, growth factors, inflammation pathway components, and transcription factors in hP-MSCs indirectly cocultured with LNCaP control or LNCaP-expressing TGF-β1. Data represent mean ± SEM. $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (Scale bars, 100 μm.)

important in stromal-epithelial interactions, including *CTGF*, *IGF1*, *BMP6*, *IL8*, *IL6*, *WNT5A*, and *WNT11* (Fig. 2*E*; *SI Appendix*, Fig. S7). Several pathway-focused PCR arrays revealed differential regulation of genes involved in a range of biological processes likely to be important in carcinoma–myofibroblast interactions (*SI Appendix*, Dataset S1). Direct-contact monolayer cocultures and 3D organoids were also analyzed to assess how cell-to-cell contact affects gene expression. Direct cocultures of RFP^{+} hP-MSCs and GFP^{+}-engineered LNCaP showed that fluorescent-activated cell sorting (FACS)-sorted hP-MSCs exhibited a similar TGF-β1–regulated gene expression profile as in the indirect cocultures (*SI Appendix*, Fig. S8). Furthermore, laser capture microdissection was used to harvest RNA from hP-MSCs in 3D organoids with TGF-β1–expressing LNCaP. hP-MSCs in 3D organoids exhibited a distinct reactive stroma gene signature (*SI Appendix*, Fig. S5*B*), consistent with the protein expression data (Fig. 2*D*; *SI Appendix*, Fig. S5*A*). In addition, PC3 human prostate cancer cells naturally express and secrete latent TGF-β1. Conditioned media from PC3 cells (TGF-β activated by acid) induced a nearly identical gene expression pattern in hP-MSCs, as observed with engineered LNCaP, and this response was abrogated by SD208, a TGF-β type I receptor blocker (*SI Appendix*, Fig. S9). Together, these results further support the concept that TGF-β1–expressing prostate cancer cells induce hP-MSCs to a myofibroblast differentiation pattern and mediate the reactive stromal response via altered expression of a wide range of growth factors and cytokines.

Transcription Factors Associated with MSC Proliferation and Myofibroblast Differentiation. To better understand mechanisms that may regulate proliferation and myofibroblast differentiation, transcription factor-focused PCR arrays were used to identify candidate genes. Gene expression analysis of hP-MSCs from indirect cocultures showed differential regulation of transcription factor expression (*SI Appendix*, Dataset S2). qRT-PCR analysis was performed to validate the top six major transcription factors upregulated by TGF-β1–expressing LNCaP in hP-MSCs (*SI Appendix*, Fig. S10). From this analysis, we further evaluated *RUNX1* and its binding partner *CBFB*, as well as *ID1*, as these exhibited higher expression levels relative to the other upregulated genes. Each of these was verified as being TGF-β1–regulated in hP-MSCs (Fig. 2*E*; *SI Appendix*, Figs. S7 and S8*B*). Consistent with increased mRNA, hP-MSCs treated with TGF-β1 also exhibited elevated expression of RUNX1 protein (*SI Appendix*, Fig. S11). RUNX1 has been identified as a key transcription factor required for definitive hematopoiesis by regulating stem/progenitor cell proliferation and differentiation (22). RUNX1 is also reported to be involved in chondrocyte differentiation (23) and was shown to be a target of TGF-β signaling (24). These findings prompted us to further study a potential functional role of RUNX1 in MSC biology and differentiation to myofibroblasts.

Inhibition of RUNX1 Promotes Myofibroblast Differentiation. Immunofluorescent staining of RUNX1 with or without TGF-β1 treatment in hP-MSCs showed nuclear RUNX1 expression only after TGF-β1 induction, suggesting that TGF-β1 induces nuclear accumulation of RUNX1 (Fig. 3*A*). To elucidate the role of RUNX1 in myofibroblast differentiation, RUNX1 expression in hP-MSCs was attenuated using two different siRNAs (Fig. 3*B*; *SI Appendix*, Figs. S12*A* and S14*A*). The prototypical genes associated with myofibroblasts, *ACTA2* and *TNC*, exhibited a dramatic increase in RUNX1 knockdown conditions (Fig. 3*D*; *SI Appendix*, Fig. S12*B*). Suppression of RUNX1 also increased *BMP6* and *PTGS2* (also known as *COX2*) but not *WNT11* (Fig. 3*D*; *SI Appendix*, Fig. S12*B*). Under coculture conditions with LNCaP expressing TGF-β1, an even greater increase in myofibroblast-associated genes was observed (Fig. 3*D*; *SI Appendix*, Fig. S12*B*). Engineered

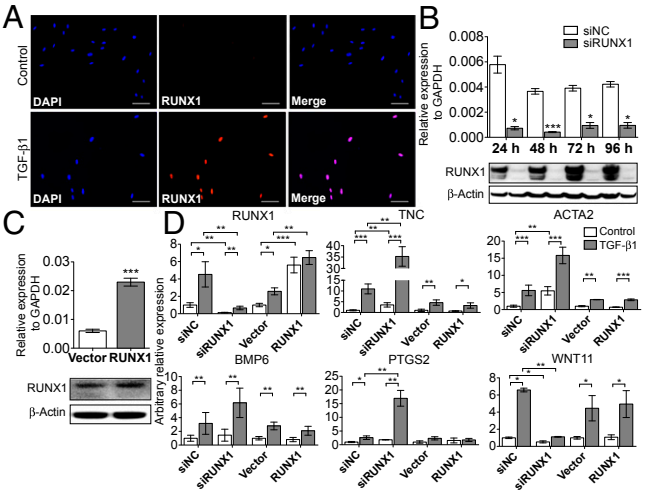

Fig. 3. Loss of RUNX1 shows distinct gene expression patterns in hP-MSCs (HPS-19I), which is potentiated by TGF-β1. (*A*) Immunofluorescent staining of RUNX1 in control and TGF-β1–treated hP-MSCs. DAPI was used to visualize cell nuclei. Note nuclear localization of RUNX1 in TGF-β1–stimulated conditions. (Scale bars, 100 μm.) (*B*) qRT-PCR and Western blot of RUNX1 in hP-MSCs transfected with control or RUNX1 siRNA. (*C*) qRT-PCR and Western blot of RUNX1 in control and RUNX1-overexpressing hP-MSCs. (*D*) Gene expression analysis in RUNX1 knockdown or overexpressing hP-MSCs cocultured with LNCaP control or LNCaP-expressing TGF-β1. Note that depletion of RUNX1 exhibited a myofibroblast signature in hP-MSCs, which was augmented by TGF-β1. Data represent mean ± SEM. $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

overexpression of RUNX1 (Fig. 3*C*) produced no significant increase or decrease in the effect of TGF-β1 on hP-MSCs (Fig. 3*D*). These results suggest that RUNX1 is permissive for myodifferentiation but may restrict complete differentiation, perhaps by maintenance of a proliferative (progenitor) status. Accordingly, we next evaluated the role of RUNX1 in mediating proliferation of MSCs.

RUNX1 Is Required for MSC Cell Cycle Progression and Proliferation. Knockdown of RUNX1 altered cell morphology. hP-MSCs with RUNX1 knockdown exhibited a more spread out, large, and flattened phenotype (Fig. 4*A*). Consistent with this phenotype, RUNX1 knockdown resulted in decreased *CD44*, *POU5F1* (also known as *OCT4*), and *BMP2* expression, yet maintained *THY1* (also known as *CD90*) expression (Fig. 4*B*; *SI Appendix*, Fig. S14*B*). THY1 is a known marker of prostate cancer-associated fibroblasts (25). Of most interest, hP-MSCs with RUNX1 knockdown were restricted from proliferation (Fig. 4*C*). Consistent with growth arrest, RUNX1 knockdown led to decrease in expression of proliferation-associated genes (Fig. 4*D*; *SI Appendix*, Fig. S14*B*). Bromodeoxyuridine (BrdU) incorporation revealed that depletion of RUNX1 resulted in a decrease in the number of cells in S phase (Fig. 4*E*; *SI Appendix*, Fig. S14*C*). Analysis of cell cycle-related gene expression showed a significant decrease in cyclin A and B, required for both S-phase progression and G2/M-phase transition and for mitosis, respectively (26–28) (Fig. 4*F*; *SI Appendix*, Fig. S14*B*). No significant differences were observed in apoptotic and necrotic cell death between control and RUNX1 knockdown hP-MSCs, as analyzed by annexin V conjugate and SYTOX Red dead cell stain (*SI Appendix*, Fig. S15). These data suggest that attenuated RUNX1 restricts proliferation by modulating cell cycle regulators without inducing cell death.

RUNX1 knockdown in marrow-derived MSCs produced nearly identical results. These cells also exhibited a more flattened phenotype and were fully growth restricted under RUNX1 knockdown conditions (Fig. 5*A*). In a similar manner, the percentage of cells in S phase was reduced (Fig. 5*E*). Expression of proliferation-associated genes and cell cycle control genes (Fig. 5 *B* and *D*), as well as differentiation/stemness-associated genes (Fig. 5*C*) exhibited a pattern consistent with those observed in hP-MSCs.

To assess how RUNX1 expression affects cell proliferative or differentiation-committed states, we examined the localization of RUNX1, Ki-67, and α-SMA after RUNX1 knockdown in both hP-MSCs and hBM-MSCs. Cell morphology changes and α-SMA expression were correlated with the nuclear intensity of RUNX1 staining (Fig. 6*A*). In control hP-MSCs, small and undifferentiated cells showed higher RUNX1 nuclear staining and lower α-SMA cytoplasmic expression (arrowheads) than large and differentiated cells (arrows). More interestingly, RUNX1-depleted hP-MSCs and hBM-MSCs revealed a myofibroblast phenotype characterized by actin polymerization, whereas controls expressed nonpolymerized actin. A similar correlation to that seen between RUNX1 and α-SMA was observed between Ki-67 and α-SMA (Fig. 6*B*). An increase in α-SMA in MSCs corresponded to loss of Ki-67 expression and morphology changes consistent with myofibroblast differentiation. Ki-67 negative cells in RUNX1 knockdown MSCs exhibited highly organized α-SMA (arrows). In addition, in vivo xenografts (day 3) constructed with LNCaP cells combined with RFP-expressing control or RUNX1 knockdown hP-MSCs also showed reduced Ki-67 staining in RUNX1 knockdown conditions (*SI Appendix*, Fig. S16). Consistent with these data, xenografts constructed with RUNX1 knockdown hP-MSCs exhibited a greater staining intensity for α-SMA (*SI Appendix*, Fig. S13). Overall, these results suggest that RUNX1 in MSCs is required for cell proliferation and modulates terminal differentiation to myofibroblasts (Fig. 6*C*).

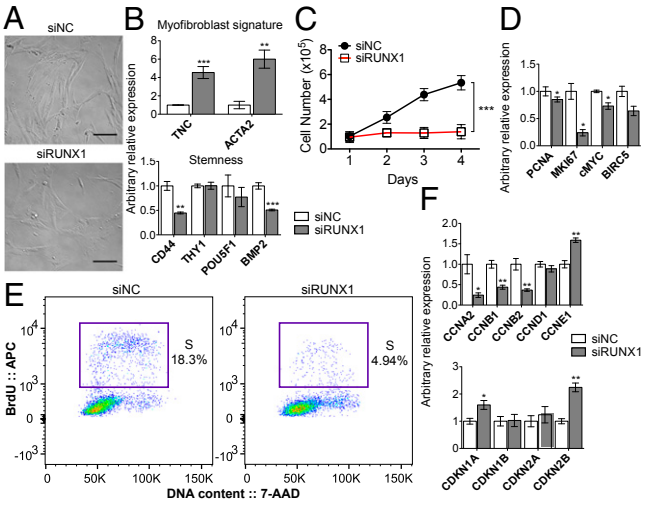

Fig. 4. Suppressing RUNX1 induces hP-MSC (HPS-19I) growth and cell cycle arrest. (*A*) Effect of RUNX1 knockdown on hP-MSC morphology. (Scale bars, 50 μm.) (*B*) qRT-PCR analysis of myofibroblast and stemness genes in control and RUNX1 knockdown hP-MSCs. (*C*) Growth curves of hP-MSCs following transfection with control or RUNX1 siRNA. (*D*) qRT-PCR analysis of proliferation-related genes in control and RUNX1 knockdown hP-MSCs. (*E*) Representative cell cycle distribution of hP-MSCs transfected with control or RUNX1 siRNA. Cells were analyzed by flow cytometry after BrdU incorporation and 7-AAD staining at 72 h posttransfection. (*F*) qRT-PCR analysis of multiple cyclins and cyclin-dependent kinase inhibitors in hP-MSCs following RUNX1 knockdown. For *B*, *D*, and *F*, data represent mean ± SEM. For *C*, data represent mean ± SD; two-way ANOVA. $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Discussion

We report the first (to our knowledge) isolation and characterization of tissue-resident MSCs derived from human normal adult prostate gland (21). The prostate-derived MSCs exhibited MSC-typical cell-surface antigen profiles and were multipotent and capable of long-term culture. Previous reports of adult human tissue-resident MSCs are restricted to dental (29), adenoid (30), and adipose (31) tissues. Importantly, we report here a critical role of RUNX1 in modulating MSC cell proliferation and committed differentiation to myofibroblasts. Myofibroblasts are stromal cells that are crucial for proper wound repair and the formation of reactive stroma associated with cancer, fibrosis, and vessel disease. Our data suggest that prostate-resident $CD44^{+}CD90^{+}$ MSCs are one potential source of reactive stroma myofibroblasts that coevolve with foci of prostate cancer and in benign prostatic hyperplasia. There are several reported sources of myofibroblasts and carcinoma associated fibroblasts in tumor-associated reactive stroma, including circulating MSCs, normal fibroblasts, circulating fibrocytes, and vessel-associated pericytes (1, 2, 32, 33). Our data do not exclude the involvement of these cell types. Our data further show that RUNX1 is an essential transcription factor for MSC proliferation and that RUNX1 modulates MSC differentiation to myofibroblasts. TGF-β1, overexpressed by prostate carcinoma cells, is a key inducer of myofibroblast differentiation and biology. We show here that RUNX1 expression is regulated by TGF-β1 in MSCs and that RUNX1 affects TGF-β1–stimulated gene expression during differentiation to myofibroblasts.

Myofibroblasts are important in normal wound closure and contribute to the pathology of several diseases and disorders. Importantly, myofibroblasts provide a prowound repair environment that forms granulation tissue and contracts to affect wound closure. The transient appearance of myofibroblasts is beneficial in restoring and maintaining tissue homeostasis. However, excessive myofibroblast proliferation and prolonged presence is detrimental for tissue function and thereby increases the risk of fibrotic diseases and cancers in many tissues including lung, liver, kidney, and prostate (4,

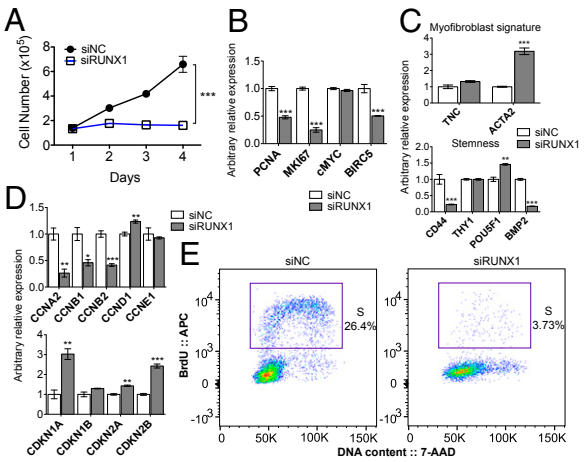

Fig. 5. Depletion of RUNX1 inhibits growth and cell cycle progression in hBM-MSCs. (*A*) Growth curve of hBM-MSCs in control and RUNX1 knockdown conditions. (*B–D*) qRT-PCR analysis in hBM-MSCs transfected with control or RUNX1 siRNA at 48 h posttransfection. Gene expression profiles of proliferation-associated genes (*B*), myofibroblast and stemness genes (*C*), and cell cycle control genes (*D*). (*E*) Representative cell cycle distribution of hBM-MSCs pulse-labeled with BrdU following transfection with control or RUNX1 siRNA for 72 h. Note that loss of RUNX1 in hBM-MSCs exhibits consistent phenotype and gene expression patterns as those shown in hP-MSCs. For *A*, data represent mean ± SD; two-way ANOVA. For *B–D*, data represent mean ± SEM. $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

34, 35). Thus, reactive stroma is associated with elevated accumulation of myofibroblasts, a hallmark of fibrosis and a predictor of cancer progression. Accordingly, identifying mechanisms that affect the genesis of myofibroblasts from precursor cells may provide potential therapeutic targets in the treatment of fibrotic diseases and cancers.

RUNX1 is a key transcription factor that regulates hematopoietic stem cells and hematopoiesis (36); however, its role in MSC and myofibroblast biology has not been reported. Our data show that RUNX1 expression is required for extended self-renewal and proliferation of MSCs and that RUNX1 expression is stimulated by TGF-β1. Attenuation of RUNX1 induced complete myofibroblast differentiation and quiescence of hP-MSCs, and this was potentiated by TGF-β1. Interestingly, these data show that TGF-β1 induces expression of genes associated with differentiation of myofibroblasts, yet also stimulates expression of RUNX1 that inhibits differentiation and maintains proliferation. The genesis of reactive stroma myofibroblasts depends on the proliferation of MSCs and their transit-amplifying progeny. Hence, one possible explanation for our data is that TGF-β1 induces MSC commitment to myofibroblast progenitor cells while maintaining proliferative status via elevated or sustained expression of RUNX1. This concept is consistent with previous reports that show that TGF-β1 stimulates proliferation of mesenchymal stem cells (37–39), progenitor cells, and several types of fibroblasts (40–43) and also induces myofibroblast differentiation and the fibrosis phenotype (44). TGF-β has been shown to stimulate fibroblast proliferation and subsequently induce their quiescence and differentiation to myofibroblasts in a coordinate manner (45). Additional factors, including CXCL12 (SDF-1), have also been shown to regulate the myofibroblast phenotype (46). TGF-β and CXCL12 pathways interact and regulate accumulation of myofibroblasts from resident fibroblasts in breast cancer (47). Hence, TGF-β1 acts in a coordinate and sequential manner to both stimulate proliferation of progenitors and induce their differentiation.

Another advance reported here was the development of a cancer cell–MSC recombined 3D in vitro organoid coculture model. This model requires no additional growth factors, serum substitutes, or extracellular matrix components. Organoid coculture of MSCs and tumor cells consistently formed a self-organizing organoid with a core of MSC-derived stromal cells surrounded by tumor cells. The 3D indirect coculture model permits a more detailed understanding of the complex mechanisms underlying carcinoma cell–stromal cell crosstalk.

Myofibroblasts are currently a potent target for developing antifibrotic therapies (48). Our findings provide insights to help identify potentially specific pathways. The overall key mechanisms and contributing cell types in the formation of reactive stroma have yet been fully identified. Perturbation of pathways that produce a biologically effective mass of myofibroblasts may offer potential for the development of antifibrotic and anticancer therapies via targeting the reactive stroma microenvironment.

Materials and Methods

Isolation of Stromal Cells from Normal Human Prostate Gland. A fresh tissue core from the peripheral zone of normal prostate was obtained from 19- and 33-y-old cadaver donors following a Baylor College of Medicine Institutional Review Board approved protocol and consents. The core was cut into discs and placed in a 96-well tissue plate containing Bfs medium [DMEM; high glucose (GIBCO) supplemented with 5% (vol/vol) FBS (HyClone), 5% (vol/vol) NuSerum (Collaborative Research), 0.5 μg/mL testosterone, 5 μg/mL insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma)]. The explants were incubated at 37 °C with 5% (vol/vol) CO_2, and medium was changed every 48 h or as necessary. Stromal cells migrated out of the explant and attached to the tissue culture dish. After the cells reached confluence, the explant was moved and the cells were passaged. Cultures at passages 7–15 were used for all experiments.

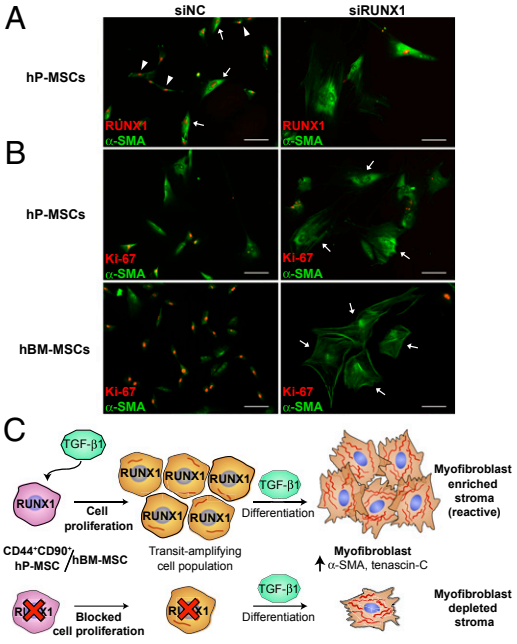

Fig. 6. The expression level of RUNX1 regulates cell proliferative or differentiation-committed state in hP-MSCs (HPS-19I) and hBM-MSCs. (*A*) Immunofluorescent staining for RUNX1 (red) and α-SMA (green) in hP-MSCs transfected with control or RUNX1 siRNA for 72 h. (Scale bars, 100 μm.) (*B*) Immunofluorescent staining for Ki-67 (red) and α-SMA (green) in hP-MSCs and hBM-MSCs with siRNA-mediated knockdown RUNX1 or control siRNA at 72 h posttransfection. Note that silencing RUNX1 increased polymerized α-SMA that corresponded to loss of Ki-67 expression and morphology changes consistent with myofibroblast differentiation (arrows). (Scale bars, 100 μm.) (*C*) Schematic diagram depicting a working hypothesis of how RUNX1 and TGF-β1 may regulate the transit amplification of MSCs to a critical mass of myofibroblasts typical of wound repair stroma and reactive stroma associated with cancer and fibrosis.

Coculture of hP-MSCs and Prostate Cancer Cells. For 3D organoid coculture, a mixture of LNCaP cells and/or either hBM-MSCs (Lonza) or hP-MSCs at different ratios was seeded onto a 12-mm Millicell-CM culture plate insert (Millipore) in a 24-well culture plate (Falcon) and incubated in LNCaP cell growth medium. After 24 h of incubation, the medium was switched to M_{20} medium [DMEM; high glucose (GIBCO) supplemented with 0.2% BSA, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (ITS; insulin, trasnsferrin, selenium supplement, Sigma)]. After 72 h of coculture, organoids were either fixed in 4% (vol/vol) paraformaldehyde for 15 min at room temperature for paraffin embedding or immediately processed for cryo-embedding. For indirect coculture, hP-MSCs (5×10^4) were seeded on a 12-mm round coverslip coated with poly-D-lysine/laminin (BD BioCoat) on a 24-well culture plate (Falcon) in Bfs medium. LNCaP or PC3 cells (4×10^5) were seeded onto a 12-mm Millicell-CM culture plate insert (Millipore) in a 24-well culture plate (Falcon) in growth medium. After 24 h of incubation, the insert containing LNCaP cells was transferred to the well containing hP-MSCs, and these cells were cocultured for 72 h in M_{20} medium. For direct coculture in monolayer, RFP-expressing hP-MSCs (1×10^6) were seeded onto a 150-mm dish (Falcon) in Bfs medium. After 24 h of incubation, an equal number of GFP-expressing engineered LNCaP cells were directly loaded onto hP-MSCs and cultured for 24 h in LNCaP cell growth medium. The next day, the medium was switched to M_{20} medium, and cells were cocultured for 72 h. hP-MSCs (RFP^+) and engineered LNCaP (GFP^+) cells from coculture experiments were suspended in Ca^{2+}/Mg^{2+}-free Dulbecco's PBS (GIBCO). RFP- and GFP-labeled cell sorting was performed with a BD FACSAria II cell sorter.

Differential Reactive Stroma Xenograft Model. Mice were injected subcutaneously with LNCaP cells and hP-MSCs with or without Matrigel as described previously (16). Xenografts were harvested after 3, 10, and 11 d.

Statistical Analysis. Student *t* test or two-way ANOVA was used to determine significance between groups. For all statistical tests, $P < 0.05$ was considered significant.

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